Contribution of TFEB-mediated autophagy to tubulointerstitial fibrosis in mice with adenine-induced chronic kidney disease

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ABSTRACT

Autophagy has been implicated in the pathogenesis of chronic kidney disease (CKD). Transcription factor EB (TFEB) is a master controller of autophagy. However, the pathophysiological roles of TFEB in modulating autophagy and tubulointerstitial injury in CKD are unknown. This study aimed to determine whether TFEB- mediated autophagy contributed to the tubulointerstitial injury in mice with CKD. After the mice were treated with an adenine diet (0.2 % adenine) for 8 weeks, the development of CKD was observed to be characterised by increased levels of plasma blood urea nitrogen (BUN), creatinine (Cre), tubulointerstitial inflammation and fibrosis. Immunohistochemical and Western blot analysis further revealed that TFEB and autophagy genes were significantly up-regulated in the kidney of the mice with adenine-induced CKD, and this increase was mostly found in the tubular epithelial cells. Interestingly, a similar expression pattern of TFEB-autophagy genes was observed in tubular epithelial cells in the kidney tissue of patients with immunoglobulin A (IgA) nephropathy. Moreover, a pathogenic role of TFEB in adenine-induced CKD was speculated because the pharmacological activation of TFEB by trehalose failed to protect mice from tubulointerstitial injuries. In the epithelioid clone of normal rat kidney cells (NRK-52E), the activation of TFEB by trehalose increased autophagy induction, cell death and inflammatory cytokine (Interleukin-6, IL-6) release. Collectively, these results suggested that the activation of TFEB-mediated autophagy might cause autophagic cell death and inflammation in tubular epithelial cells, contributing to renal fibrosis in adenine-induced CKD. This study provided novel insights into the pathogenic role of TFEB in CKD associated with a high purine diet.

1. Introduction

Chronic kidney disease (CKD), a condition with progressive and irreversible losing kidney function characterised by glomerular injury and tubulointerstitial fibrosis, is a global health problem with a prevalence of 5 %–10 % of the world population [1]. Although studies on the molecular mechanisms and signalling pathways of CKD have progressed recent decades, the event that initiates this process is still unclear. Studies have confirmed that CKD has a close relationship with renal interstitial fibrosis which generally refers to the excessive deposition of extracellular matrix (ECM) components in tubulointerstitium [2]. Fibrogenic mechanisms are primitively for repairing the short-term injury. During kidney fibrosis, collagen-rich scar tissue progressively accumulates in the interstitial space. Persistent injury results in scar accumulation, which disrupts the normal function of tubular epithelial cells. The contractibility of the scar fibres further facilitates tissue dysfunction and advances renal fibrosis toward end-stage renal failure [3]. Therefore, slowing down fibrosis progression or even accelerating its reversion can be a therapeutic strategy for CKD.

Autophagy has been extensively studied and presented to be a critical role in fibrosis-associated CKD. It is an evolutionarily conserved bulk degradation system in eukaryotes and may control the intracellular environment and energy status by eliminating the abnormal proteins and organelles [4]. However, the role of autophagy in kidney fibrogenic response remains controversial. On the one hand, autophagy activation can rescue the inhibition of the G2/M arrest of the cell cycle and the subsequent fibrogenic response in angiotensin II-stimulated renal epithelial cells and unilateral ureteral obstruction model of mice [5]. On the other hand, autophagy can promote lipid droplet accumulation in tubular epithelial cells, which subsequently cause renal lipotoxicity and carry forward the progression of kidney fibrosis [6]. And persistent activation of autophagy in the proximal tubules of the kidney promotes renal interstitial fibrosis during unilateral ureteral obstruction (UUO) by regulating tubular cell death, interstitial inflammation, and profibrotic factor production [2].

TFEB is the master regulator of lysosomal biogenesis and autophagy. Under normal physiological conditions, TFEB is phosphorylated by mammalian target of the rapamycin C1 (mTORC1) which results in its reservation in the cytoplasm. Under starvation or other stress conditions, mTORC1 is released from the lysosomes, consequently, TFEB becomes dephosphorylated, resulting in rapid nuclear translocation of TFEB, its binding to the promoter regions of autophagy related genes, and its induction of autophagosome biogenesis and autophagosome- lysosome fusion [7]. Studies on TFEB-mediated autophagy in renal diseases have mainly focused in renal carcinoma [8–10], but have rarely reported about TFEB-mediated autophagy and renal fibrosis. Despite the emerging evidence

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suggesting that autophagy occurs during renal interstitial fibrosis, the role of autophagy activation in fibrosis and the mechanism on how autophagy influences fibrosis remain controversial. In the present study, the role of TFEB-mediated autophagy in CKD-related fibrosis and the underlying mechanism were investigated.

2. Materials and methods

2.1. Animals

Wild-tpye C57BL/6 J male mice were obtained from the Model Animal Research Center of Nanjing University (Nanjing, China). Mice were maintained on standard laboratory condition with room temperature (22 ± 2 ℃) under an alternating 12 h light-dark cycle. And they had access to food and water ad libitum. In the first set of animal experiments to investigate the change of TFEB expression level on adenine diet induced CKD, after an acclimatization period of seven days, mice were fed with normal diet or normal diet supplemented with 0.2 % adenine for 8 weeks. During the treatment periods, animals were weighed weekly. At the end of the treatment the animals were anesthetized with an intraperitoneal injection of pentobarbital sodium (75 mg/kg). Kidney samples were then rapidly removed.

In the further animal experiments, to investigate the effect of TFEB activation on adenine-induced CKD, mice were randomly divided into 3 groups, a third of the mice fed with normal diet and two third of the mice fed with adeine diet for 6 weeks. Among them, a half of mice which fed with adenine diet were intraperitoneal injected 2 mg/g trehalose in PBS for 5 times per week at the same time. Subsequent processing is the same as before.

2.2. Biochemical parameters of plasma

Blood samples were collected from the retroorbital sinus under anesthesia. Plasma was obtained after 3000 rpm centrifugation for 15 min at 4 ℃. The levels of BUN, Cre, Ca^{2+} and Pi in plasma were measured using biochemical kit (Nanjing Jiancheng, China) following the manufacturer's protocols.

2.3. Histology analyses

The kidney tissues were fixed with 4% paraformaldehyde for at least 24 h. The samples were then dehydrated in ethanols and embedded in paraffin. 4-μm kidney paraffin sections were further processed for Hematoxylin&Eosin (H&E) staining (Nanjing Jiancheng, China), Masson's trichrome staining (Nanjing Jiancheng, China) according to the manufacturer's instructions. *2.4. Immunohistochemistry staining*

After deparaffinization, hydration and antigene retrieval in 0.01 M citrate buffer (pH = 6.0), 4-μm kidney paraffin slices were washed with 3 % H_2O_2 for 10 min, followed by blocked in 5 % goat serum and incubated overnight with the primary antibodies F4/80 (1:250, CST, USA), Nlrp3 (1:50, Abcam, USA), αSMA (1:1000, CST, USA), TFEB (1:1000, Bethyl, USA), LC3 (1:500, CST, USA), Beclin1 (1:50, CST, USA), p62 (1:150, Abcam, USA), then incubation with the corresponding secondary antibodies (1:200, Vector, USA) and HRP (1:200, Bostor, China) for two 30 min. DAB (Vector, USA) was applied for 2 min. Sections were counterstained with hematoxylin for staining cell nucleus. After permeabilizing, the tissue section was preserved by mounting solution. *2.5. Electron microscopy*

For ultrastructural analysis kidney cortices were fixed in 4 % paraformaldehyde and 1 % glutaraldehyde in 0.1 M PB, dehydrated, permeated, embedded, sliced on a microtome (60-nm, Leica, Germany) and stained with uranyl acetate overnight. The ultrathin sections were analysed using the HT7700 transmission electron microscope (HITACHI, Japan) to detect autophagosomes or autolysosomes.

2.6. Cell culture

NRK-52E cells, the epithelioid clone of the normal rat kidney cells, were obtained from *Procell Life Science&Technology Co,.Ltd*. Cells were grown in Dulbecco's Modified Eagle Medium (4.5 g/L D-glucose, Gibco) containing 10 % fetal bovine serum (FBS) and 1 % penicillin- streptomycin (Gibco, USA) at 37 ℃ under a humidified atmosphere of 5 % CO_2. At >90 % confluence, cells were trypsinized and seeded onto 6- well plates (2×10^5/ well). After 24 h, cells were changed fresh contained 0.5 % FBS complete medium for 1 h, then added trehalose (Sigma, USA) to the final concentration of 0, 10, 50, 100 mM for futher 48 h. In the autophagy inhibitor assay, 50 nM bafilomycin (Selleckchem, USA), 40 μM chloroquine (Sigma, USA) and 1 mM 3-MA (TOCRIS, UK) were pretreated cells for 2 h, then changed 100 mM trehalose for further 12 h.

2.7. Confocal microscopy

The fluorescence images were collected using a scanning confocal microscope (ZEISS, Germany). Cells were cultured on 35-mm on glass coverslips and treated with or without 100 mM trehalose for 6 h. Cells were then fixed with 4 % paraformaldehyde for 15 min, and permeabilized with 0.5 % Triton X-100 for 20 min. Following 30 min incubation in blocking 5 % donkey serum, cells were incubated with rabbit anti-TFEB (1:500, Vector, USA) overnight at 4 ℃. Then, cells were washed and stained with 488 donkey anti-rabbit secondary antibody (1:500, Invitrogen, USA) for 1 h. Finally, cells were mounted using vectamout permanent mounting medium (Vector, USA) containing DAPI (Sigma, USA). The effect of trehalose on TFEB was determined by staining with TFEB antibody and DAPI to assessed TFEBs' nuclear translocation. *2.8. Western blot*

Kidney tissues and cells were lysed directly on ice with RIPA buffer (GBCBIO, China) supplemented with protease inhibitor (Roche, USA) to obtained total protein. Protein concentration was quantified by a commercial BCA protein assay kit (Beyotime, China). The equivalent of 25 μg of protein was loaded onto the gel for 12 % or 15 % SDS-PAGE and transferred onto a PVDF membrane (Millipore, USA). Then the membrane was blocked in 5 % non-fat milk for 1.5 h at room temperature, followed by incubated at 4 ℃ overnight with the primary antibodies specific to

TFEB (1:5000, Vector, USA), LC3A/B (1:1000, CST, USA), Beclin1 (1:1000, CST, USA), p62 (1:2000, Abcam, USA), Bcl-2 (1:1000, Affinity, USA), caspase3 (1:1000, GeneTex, USA), αSMA (1:1000, CST, USA), GAPDH (1:1000, CST, USA), β-actin (1:1000, Bostor, China). On the next day, the PVDF membrane was immersed in the corresponding secondary antibody for 1.5 h. Protein bands on the membrane were visualized with ECL kit (Millipore, USA). Band intensities were quantitated using ImageJ software.

2.9. Quantitative RT-PCR

Total RNA was extracted from NRK-52E cells using RNAiso Plus (Takara, Japan) according to the manufacturer's instructions. RNA concentration and purity were detected by nucleic acid quantitative determination meter (Thermo, USA). The total RNA was reverse transcribed by using PrimeScript™ RT reagent Kit (Takara, Japan). The oligonucleotide primers were as follow, IL-6: Forward 5′-GTCAACTCCATCTGCCCTTCAG-3′, Reverse 5′-CTCCTGGTATGAAGTGGCAAATC-3′ [11]; GAPDH: Forward 5′-TGACAACTTTGGCATCGTGG-3′, Reverse 5′-GGGCCATCCACAGTCTTCTG-3′ [12]. GAPDH was used as the endogenous control. The relative gene expression was determined through Real-Time PCR system (Bio-Rad, Singapore) by using SYBR Premix Ex Taq™ (Takara, Japan) according to the manufacturer's recommended protocol.

2.10. Statistical analysis

Results are expressed as mean ± SEM. Differences between groups were assessed by the *t*-test or ANOVA using SPSS 19.0 software, and *p* values <0.05 were considered statistically significant.

3. Results

3.1. Adenine diet-induced renal injury is associated with tubulointersitial inflammation and fibrosis

Mice were treated with an adenine-rich diet (0.2 % adenine) for 8 weeks to establish CKD model. In accordance with previous studies, the mice treated with the adenine-rich diet exhibited weight loss as early as 2 weeks, and no mice died until treatment for 5 weeks (Fig. 1A). The biochemical findings revealed that the increased mortality in the adenine diet-treated mice was associated with severe renal dysfunction. Fig. 1**B-C** depict the concentrations of plasma BUN and creatine in the mice treated with adenine for 2–8 weeks. BUN and creatinine significantly increased in the adenine diet-treated mice as early as 2 weeks. CKD is commonly associated with calcium and phosphorus metabolism disorders. Moreover, the adenine diet-fed mice exhibited significantly increased in plasma calcium (Ca^{2+}) and phosphate (Pi) levels (Fig. 1**D- E**). The urine volume of the mice with adenine diet was much higher than the normal diet fed mice (data not shown), which might be due to adenine-induced damage in the interstitial tubular layer that impairs water resorption.

In Fig. 1F, H&E staining showed that the adenine-fed mice exhibited obvious tubular injury, with a higher prevalence of the dropout of tubular epithelial cells, especially in proximal tubules and tubular luminal expansion. Nlrp3 inflammasome is a critical intercellular machinery to trigger tissue inflammation in several diseases, including CKD [13–15]. In the kidney of adenine mice, injured renal tubules showed an obviously positive Nlrp3 expression (Fig. 1**G-H**). Fig. 1**I-J** show that the normal kidney had no F4/80 positive cells in the control mice, whereas F4/80 positive cells accumulated in the tubular interstitium of the kidney of the adenine diet-fed mice. These data suggested that epithelial cells injury might facilitate the invasion of immune cells, such as macrophages in the tubulointerstitium, leading to tubulointerstitial inflammation in these mice. Moreover, Masson's trichrome staining (Fig. 1K) demonstrated that such tubulointerstitial inflammation was accompanied with the massive interstitial deposition of collagens. Therefore, tubulointerstitial fibrosis was induced in the kidneys of adenine mice.

Female mice were also used for adenine-induced CKD (Supplementary Fig. 1). The female mice also showed weight loss, increased plasma creatine and BUN levels and tubulointerstitial inflammation and fibrosis. However, they were more tolerant of adenine diet than the males as indicated by the higher survival rate and the later onset of renal injury upon adenine diet treatment.

3.2. TFEB-mediated autophagy is persistently induced in renal tubular epithelial cells in adenine-treated mice

In this study, whether TFEB-autophagy is involved in the development of renal dysfunction was investigated. The distribution of TFEB and its expression in the tissue sections of kidneys were examined through IHC. Fig. 2**A-B** show the non-detectable or minimal staining of TFEB in the kidney of the control mice, whereas the TFEB staining intensity significantly increased in the adenine diet-treated mice, particularly in proximal tubules. This increase in the TFEB expression in proximal tubules was accompanied with the increase in Beclin-1 (Fig. 2**C-D**), which is an autophagy activator inducing phagophore formation, and LC3 (Fig. 2**E-F**), which is an autophagosome membrane- associated protein. However, the selective autophagosomal substrate p62 had no obvious change (Fig. 2**G-H**). Western blot was also used to quantitatively analyse the relevant protein expression, as shown in Supplementary Fig. 2**A-D.** These findings were consistent with the corresponding IHC results. Electron microscopy was conducted to detect autophagosomes or autolysosomes (Fig. 2**I**) to further confirm autophagy in adenine-treated mice. The number of single membrane- surrounded autolysosomes with some undigested organelles or proteins increased in AD-treated mice. Therefore, adenine treatment triggered the TFEB-mediated autophagy signalling pathway in the kidney, primarily in proximal tubules.

3.3. TFEB-mediated autophagy is upregulated in renal tubules in humans with CKD

The human renal biopsy samples from nine patients with different degrees of IgA nephropathy were analysed to further confirm the correlation

between TFEB-mediated autophagy and renal fibrosis. IgA nephropathy is a primary glomerular nephropathy. The original renal biopsies were reviewed by two pathologists and scored using the Oxford Classification of IgAN (MEST score). The level of tubular atrophy/ interstitial fibrosis was divided into T0 ≤ 25 %, T1 25 %–50 % and T2 ≥ 50 %. As illustrated in Fig. 3, TFEB and LC3 expression levels increased in tubular epithelial cells in patients with T2 degree compared with those in patients with T0 degree, and these increaseed levels in TFEB and LC3 were accompanied with the up-regulation of the αSMA expression in the tubulointerstitium.

3.4. Effects of TFEB activation by trehalsoe on adenine-induced fibrosis

In this study, whether renal dysfunction could be prevented or ameliorated by trehalose, a pharmacological TFEB activator as reported in many other studies, was examined to investigate the possible protective effect of TFEB in the development of adenine-induced renal dysfunction. The related animal experiment flow chart is shown in Fig. 4**A**. In Fig. 4**B-D**, the mice treated with adenine plus trehalose concomitantly showed similar weight loss and increased plasma BUN and creatinine levels. Furthermore, trehalose did not prevent adenine-induced kidney injury as trehalose-treated mice had no improvement in the histological appearance compared with that in the adenine-

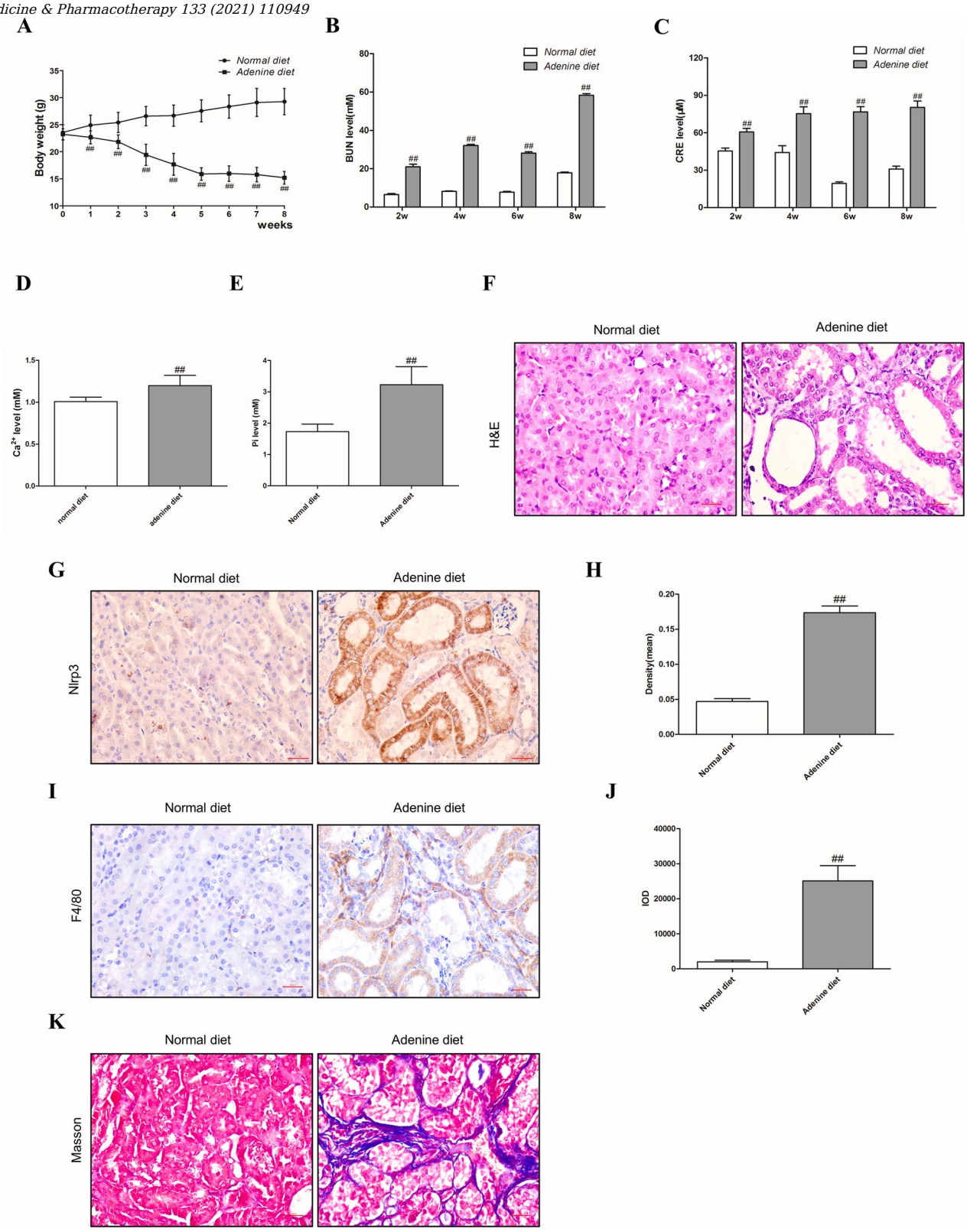


Fig. 1. Adenine diet-induced renal injury is associated with tubulointersitial inflammation and fibrosis. **A.** Bodyweight (n = 7~9). **B.** BUN level (n = 7~9). **C.** Cre level (n = 7~9). **D.** Ca^{2+} level (n = 7~9). **E.** Pi level (n = 7~9). **F.** Histological characteristics of representative kidney sections stained with Hematoxylin & Eosin (400×). Scale bar = 100 μm. **G.** Representative kidney immunohistochemical staining of Nlrp3 (400×), the dark brown areas show increased tubular epithelial cell inflammation areas. Scale bar = 100 μm. **H.** Summary of Nlrp3 immunohistochemical staining results (n = 6). **I.** Representative kidney immunohistochemical staining of F4/80 (400×), the dark brown areas show increased renal interstitial immune cell infiltration areas. Scale bar = 100 μm. **J.** Summary of F4/80 immunohistochemical staining results (n = 6). **K.** Histological characteristics of representative kidney sections stained with Masson (400×), blue staining identified the regions of collagen fiber hyperplasia, indicative of fibrotic changes. Scale bar = 100 μm. All data are presented as mean ± S.E.M. #

means $p < 0.05$, ## means $p < 0.01$ vs. normal diet group. (For interpretation of the references to colour in the Figure, the reader is referred to the web version of this article).

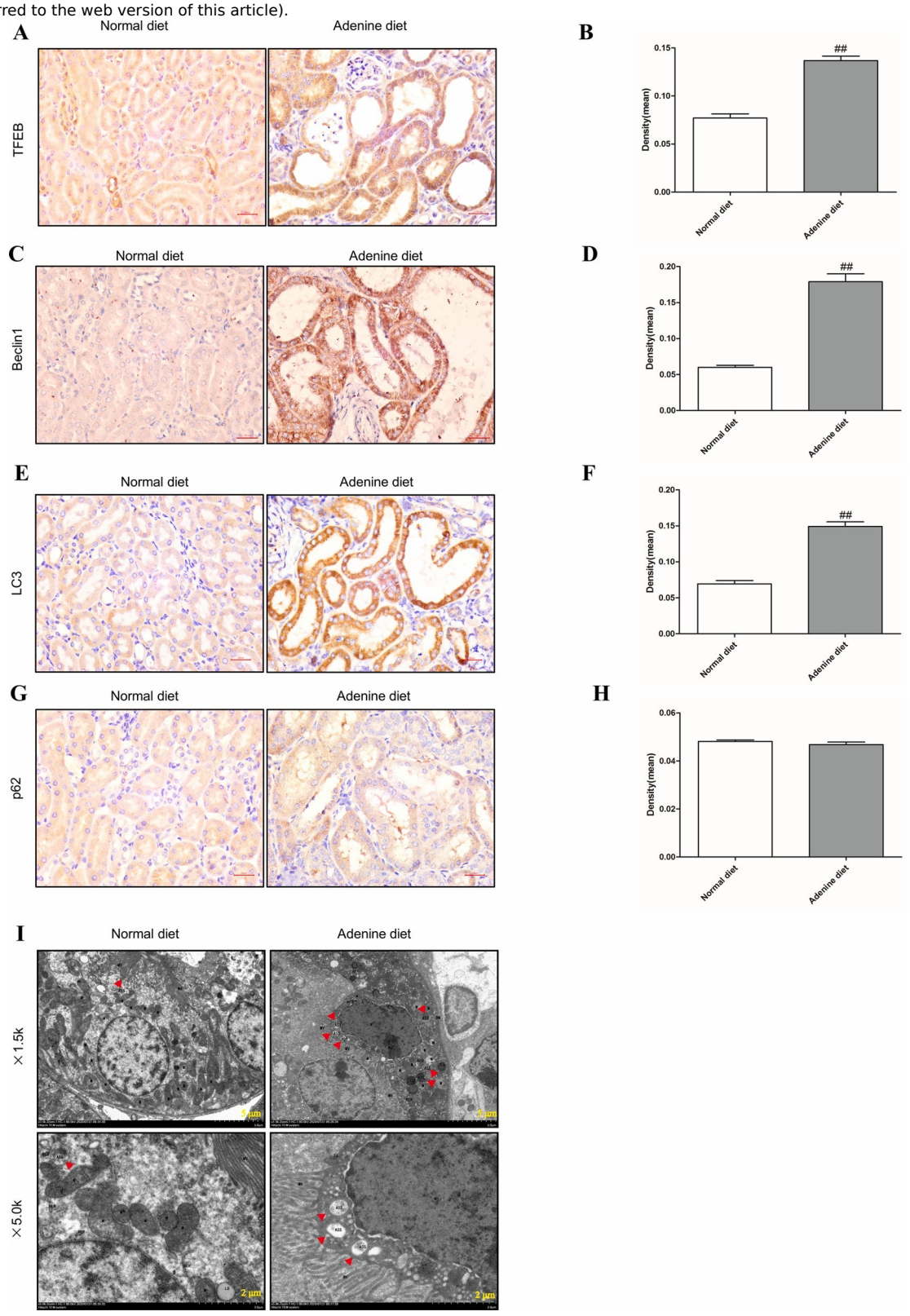


Fig. 2. TFEB-mediated autophagy is persistently induced in renal tubular epithelial cells in AD-treated mice. **A.** Representative kidney immunohistochemical staining of TFEB (400×). Scale bar = 100 μm. **B.** Summary of TFEB immunohistochemical staining results (n = 6). **C.** Representative kidney immunohistochemical staining of Beclin1 (400×). Scale bar = 100 μm. **D.** Summary of Beclin1 immunohistochemical staining results (n = 6). **E.** Representative kidney immunohistochemical staining of LC3 (400×). Scale bar = 100 μm. **F.** Summary of LC3 immunohistochemical staining results (n = 6). **G.** Representative kidney immunohistochemical staining of p62 (400×). Scale bar = 100 μm. **H.** Summary of p62 immunohistochemical staining results (n = 6). **I.** Utrastructural analysis revealed increase of autolysosomes in proximal tubular cells in AD-treated mice. Autolysosomes are marked with “ASS” exemplarily, mitochondria are marked with “M”, lipid droplet is marked with “LD”,

nucleus is marked with “N”, lysosome is marked with “Ly”, rough endoplasmic reticulum is marked with “RER”, microvilli is marked with “MV”, tight junction is marked with “TJ”. × 1.5k, scale bar =5 μm, ×5.0k, scale bar =2 μm. All data are presented as mean ± S.E.M. $^{\#}$ means $p < 0.05$, $^{\#\#}$ means $p < 0.01$ vs. normal diet group.

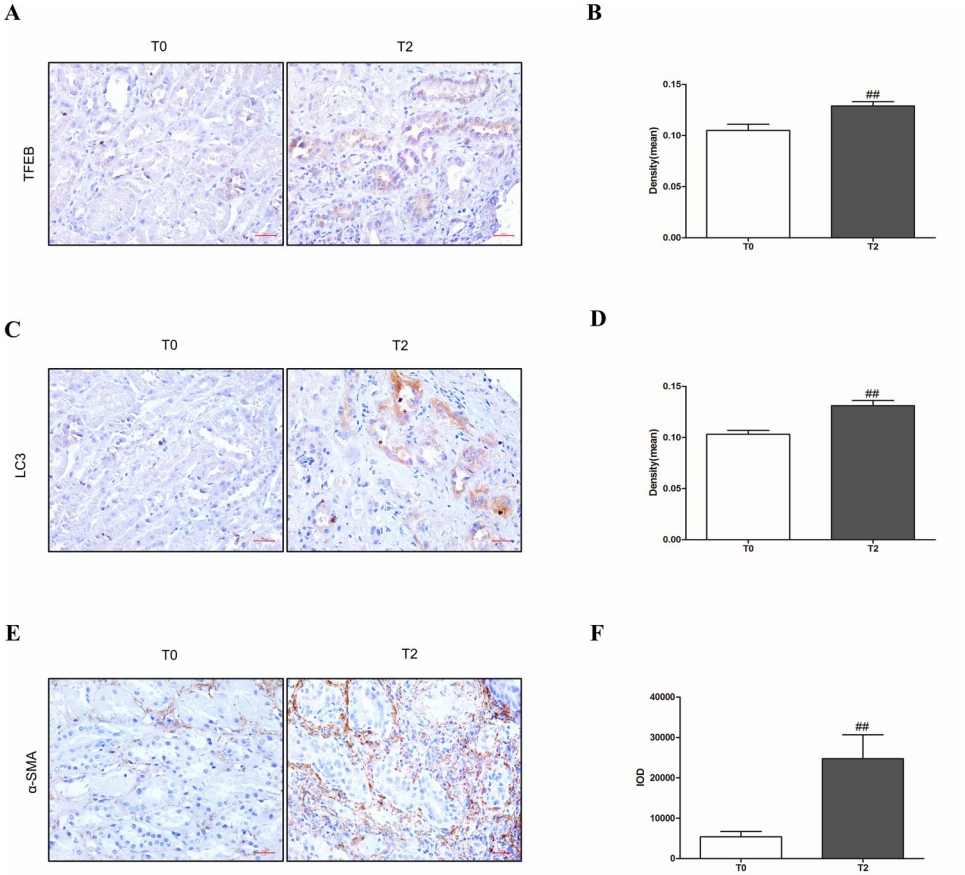


Fig. 3. TFEB-mediated autophagy is upregulated in renal tubules in humans with CKD. **A.** Representative kidney immunohistochemical staining of TFEB (400×). Scale bar = 100 μm. **B.** Summary of TFEB immunohistochemical staining results (n = 4~5). **C.** Representative kidney immunohistochemical staining of LC3 (400×). Scale bar = 100 μm. **D.** Summary of LC3 immunohistochemical staining results (n = 4). **E.** Representative kidney immunohistochemical staining of αSMA (400×). Scale bar = 100 μm. **F.** Summary of αSMA immunohistochemical staining results (n = 4~5). All data are presented as mean ± S.E.M. $^{\#}$ means $p < 0.05$, $^{\#\#}$ means $p < 0.01$ vs. the patients with T0 degree.

treated groups with a similar dilation of the tubules, interstitial inflammatory cell infiltration and tubular atrophy (Fig. 4E). The trehalose treatment also had no effects on tubulointerstitial inflammation and fibrosis as shown by F4/80 (Fig. 4**F-4G**) and αSMA (Fig. 4**H-I**). The expression levels of TFEB in the proximal tubules of trehalose-treated mice were higher than those in the mice fed with adenine diet alone (Fig. 4**J-K**). the αSMA and TFEB protein expression levels were also confirmed through Western blot (Supplementary Fig. 2**E-F)**. These data excluded the possibility that TFEB activation could protect tubulointerstitial inflammation and fibrosis in adenine-treated mice. However, the role of TFEB should be further investigated in adenine-induced CKD.

3.5. Activation of TFEB induces autophagy in the epithelioid clone of the normal rat kidney cells

Given that adenine-induced TFEB activation primarily occurred in renal proximal tubules, we then examined whether TFEB-mediate autophagy signalling was functioning in the epithelioid clone of the normal rat kidney cells (NRK-52E cells). As shown in Fig. 5A, the treatment of NRK-52E cells with trehalose significantly increased the nuclear translocation of TFEB as shown by the enhanced colocalisation of TFEB and DAPI. Accompanied with the translocation of TFEB into the nuclei, the cytoplasmic expression of TFEB was decreased by trehalose in a dose-dependent manner (Figs. 5**B-5C**). We also detected the TFEB expression in the nuclear fraction, but we found that its expression was too low to be analysed. Consistent with the TFEB activation, trehalose increased the expression levels of Beclin1 (Fig. 5**D-E**), LC3 (Fig. 5**F-H**), and p62 (Fig. 5**I-J**). Hence, the activation of TFEB by trehalose could directly induce autophagy signalling in NRK-52E cells.

3.6. Activation of TFEB induces autophagic cell death and inflammation in the epithelioid clone of the normal rat kidney cells

The excessive induction of autophagy may cause an imbalance of autophagosome generation and degradation, leading to autophagic cell death. The pro-apoptotic and pro-inflammatory effects of trehalose on NRK-52E cells were examined. As illustrated in Fig. 6A, trehalose markedly decreased the viability of NRK-52E cells in a dose-dependent manner with a maximal reduction by 50 % at 100 mM concentration. Consistently, trehalose decreased the expression of the anti-apoptotic protein Bcl-2 (Fig. 6**B-C**) and increased the activation of caspase-3 (Fig. 6**D-E**). In addition, trehalose increased the mRNA level of IL-6

(Fig. 6F), a typical inflammatory cytokine associated with tubulointerstitial inflammation. The mRNA level of another cytokine TNF-α was also measured (data not shown), but its expression was extremely low to be analysed. Three autophagy inhibitor bafilomycin, choloquine and 3- MA, were used to further confirm whether trehalose-induced cell death was associated with autophagy (Fig. 6G**)**, but only bafilomycin could block trehalose-induced cell death.

4. Discussion

This study aimed to determine whether TFEB-mediated autophagy signalling played a modulatory role in renal dysfunction in a mouse model of CKD. Our results demonstrated that TFEB had an unprotected role in the development of renal dysfunction in mice with adenine- induced CKD, which was attributed to the triggering role of TFEB in promoting autophagic cell death in renal tubular epithelial cells and consequent tubulointerstitial inflammation and fibrosis.

Recent studies have established a noninvasive mouse model of CKD by feeding mice with an adenine-rich diet [16,17]. The mechanisms involved in adenine-induced renal injury include mechanical stretch

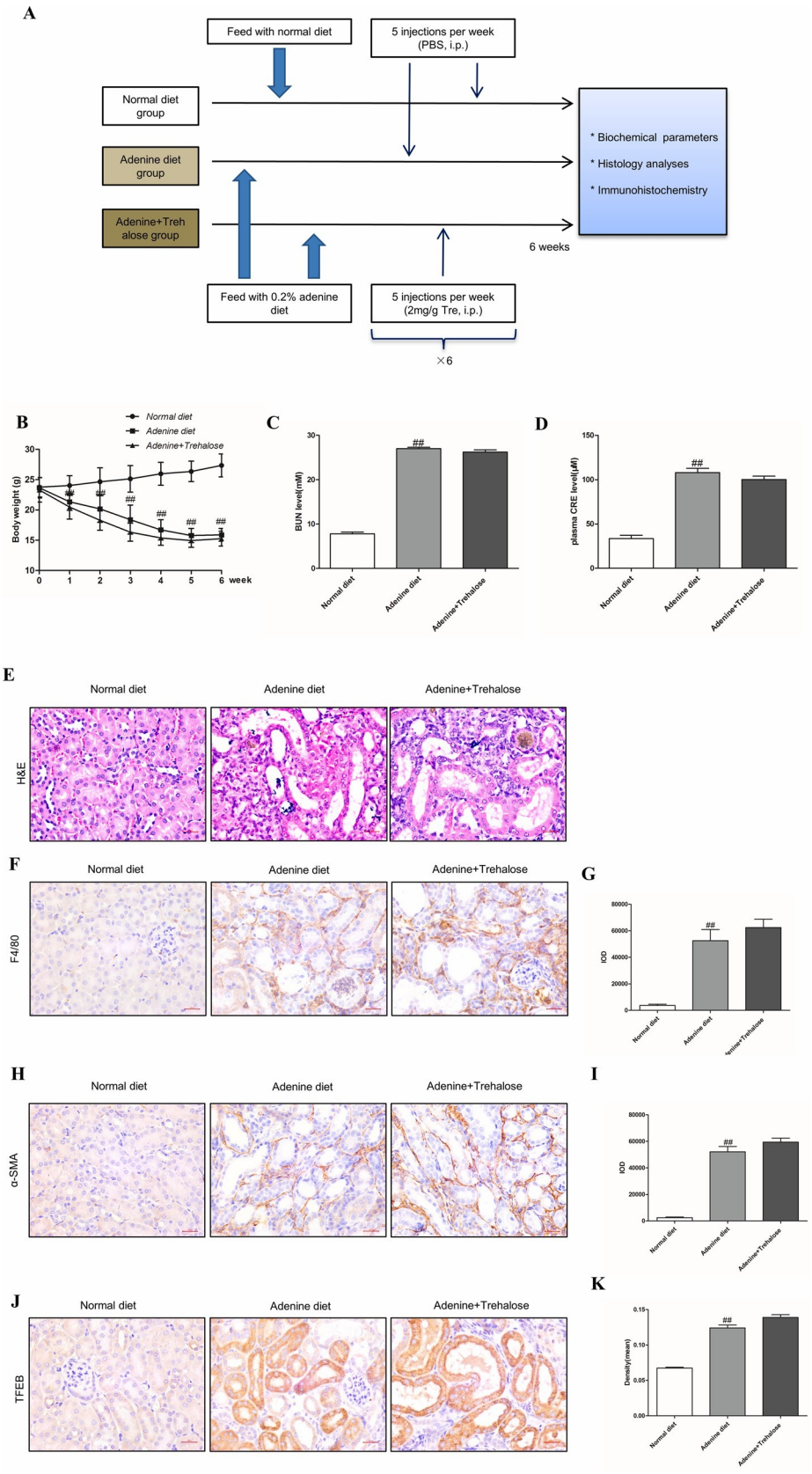


Fig. 4. Effects of TFEB activation by trehalsoe on AD-induced fibrosis. **A.** Diagram summarizing experimental protocol and mice cohorts used for *in vivo* assessment of the effect of trehalose in AD-induced renal injury. Mice were fed with 0.2 % adenine diet and being administered either PBS, trehalose (given i.p. 2 mg/g for five times per week) for 6 weeks. **B.** Bodyweight (n = 6~8). **C.** BUN level (n = 6~7). **D.** Cre level (n = 6~7). **E.** Histological characteristics of representative kidney sections stained with Hematoxylin & Eosin (400×). Scale bar = 100 μm. **F.** Representative kidney immunohistochemical staining of F4/80 (400×), the dark brown areas show increased renal interstitial immune cell infiltration areas. Scale bar = 100 μm. **G.** Summary of F4/80 immunohistochemical staining results (n = 6). **H.** Representative kidney immunohistochemical staining of αSMA (400×), the dark brown areas show increased renal interstitial fibrosis areas. Scale bar = 100 μm. **I.** Summary of αSMA immunohistochemical staining results (n = 6). **J.** Representative kidney immunohistochemical staining of TFEB (400×). Scale bar = 100 μm. **K.** Summary of TFEB immunohistochemical staining results (n = 6). All data are presented as mean ± S.E.M. $^{\#}$ means $p < 0.05$, $^{\#\#}$ means $p < 0.01$ vs. normal diet group.

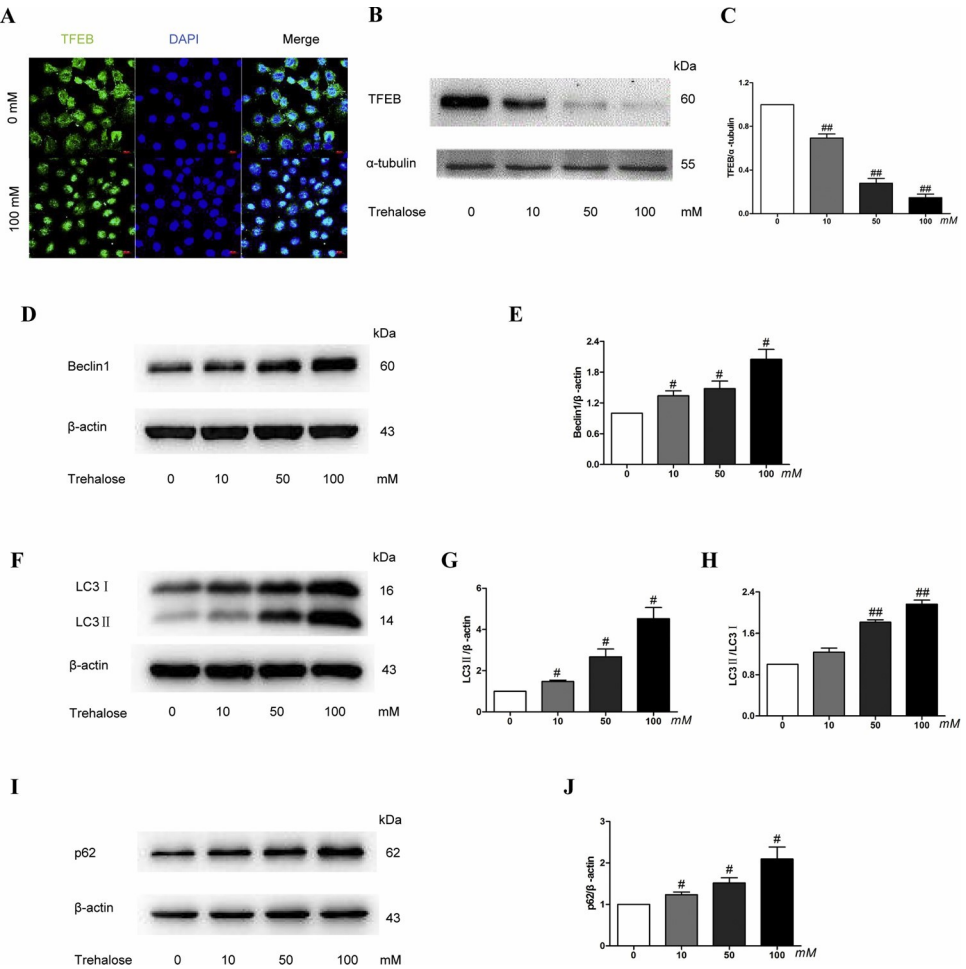
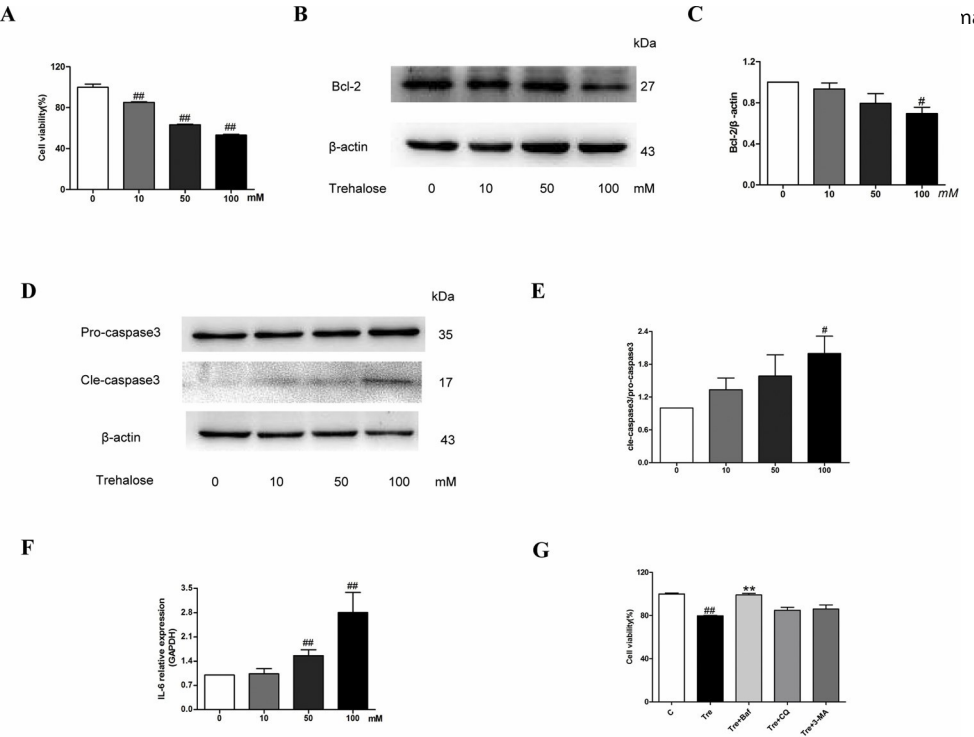


Fig. 5. Activation of TFEB induces autophagy in the epithelioid clone of the normal rat kidney cells. **A.** Fluorescence images indicate the effect of trehalose on the co-localization of TFEB (green) with DAPI (blue) in NRK-52E cells. **B.** Western blot analysis shows the effect of trehalose on TFEB expression in cytosol. **C.** Summary of TFEB Western blot results (n = 3). **D.** Western blot analysis shows the effect of trehalose on Beclin1 expression. **E.** Summary of Beclin1 Western blot results (n = 3). **F.** Western blot analysis shows the effect of trehalose on LC3 expression. **G and H.** Summary of LC3 Western blot results (n = 3). **I.** Western blot analysis shows the effect of trehalose on p62 expression. **J.** Summary of p62 Western blot results (n = 3). All data are presented as mean ± S.E.M. $^{\#}$ means $p < 0.05$, $^{\#\#}$ means $p < 0.01$ vs. 0 mM trehalose. (For interpretation of the references to colour in the Figure, the reader is referred to the web version of this article).



nal rat kidney cells A. MTT analysis shows the effect of trehalose on the viability of NRK-52E cells (n = 6). **B.** Western blot analysis shows the effect of trehalose on Bcl-2 expression. **C.** Summary of Bcl-2 Western blot results (n = 3). **D.** Western blot analysis shows the effect of trehalose on caspase3 expression. **E.** Summary of caspase3 Western blot results (n = 3). **F.** q- PCR analysis shows the effect of trehalose on IL- 6 mRNA level (n = 8). **G.** MTT analysis shows the effect of autophagy inhibitor on trehalose- induced cell death (n = 6). All data are presented as mean ± S.E.M. $^{\#}$means $p < 0.05$, $^{\#\#}$ means $p < 0.01$ vs. 0 mM trehalose, * means $p < 0.05$, ** means $p < 0.01$ vs. 100 mM trehalose.

from urine accumulation and tubular distension, oxidative stress, hypoxia and pro-inflammatory and pro-fibrotic factors [16]. Consistent with previous findings [16], our results revealed that mice with adenine diet exhibited a significant weight loss as early as 1 week after adenine treatment. The survival rate of the mice decreased after 5 weeks of treatment. The weight loss and death indicated a progressive development of morbidity and mortality in adenine-treated mice. This study also found that renal dysfunction occurred as early as 2 weeks after adenine treatment as shown by the increased levels of BUN and serum creatinine. Our immunohistological studies showed that adenine induced marked tubular and interstitial injuries and metabolic abnormalities in the mice as characterised by tubular atrophy and dilatation, glomerular capillary collapse, interstitial inflammation and fibrosis, which resemble chronic renal failure in humans [16,17]. Our findings were consistent with recent results showing that male C57BL/6 J mice are sensitive to renal injury after they receive an adenine-rich diet [16,17]. Moreover, we noticed a previously unreported sex difference in the progression of adenine-induced renal dysfunction and injury in mice. The female mice on adenine diet exhibited less weight loss, prolonged survival and delayed onset of renal glomeruli and tubular damages. However, studies have yet to determine whether the protective effect of oestrogen in the kidney gives rise to sex difference. Nonetheless, our findings supported the view that the adenine model simulates the human renal disease in its natural course with similar histological features. Therefore, understanding the precise molecular mechanisms underlying renal damage in adenine-induced CKD and using this model to predict renal functional outcome and responsiveness to pharmacological interventions have potential clinical implications.

Accumulating evidence indicates that autophagy plays dual roles that can be either protective or injurious in various animal models of renal dysfunction or CKD [18–20]. A recent study showing that adenine induces autophagy and renal tubular injury in rats [20]; however, the cause–effect relationship of autophagy, tubular injury and related molecular mechanisms for autophagy induction has not been investigated. TFEB is a master controller of autophagy signalling in a variety of mammalian cells by up-regulating autophagy-related genes, including Beclin1, LC3, p62 and ATG9B, and its overexpression in cultured cells increases the formation of autophagosomes [21]. Thus far, the role of TFEB in renal dysfunction and CKD remains unknown. Here, we demonstrated that the TFEB expression increased mostly in renal tubules in adenine-treated mice, and autophagy signalling enhanced, as indicated by the increased expression levels of LC3 and Beclin1. The enhanced autophagy usually leads to the degradation of the autophagic substrate p62. However, in the present study, adenine diet did not reduce the p62 expression in the renal tubules. This phenomenon might be due to the transcriptional up-regulation of p62, a downstream target gene of TFEB transcription factor activity [22,23]. We also examined autolysosome through electron microscopy to confirm the increased autophagy level in adenine diet-treated mice. To the best of our knowledge, this study provided the first evidence that TFEB was involved in autophagy signalling in the renal tubules. This phenomenon was consistent with the finding that the LC3 expression was up-regulated in the renal tubules of adenine-treated rats [20]. The present study also demonstrated that the expression levels of TFEB and LC3 were positively correlated with the severity of fibrosis in human kidney biopsies (Fig. 3). Consistently, previous studies showed that the increased autophagic activity is found in tubular cells in human biopsy samples from the transplanted kidney [18] and patients with nephropathic cystinosis [24]. These findings indicated that TFEB-autophagy signalling activation in the pathogenesis of renal tubular injuries and CKD in animals and humans. Thus, we further investigated the role of TFEB activation in CKD progression.

Trehalose is a pharmacological activator of TFEB, which has been widely used in TFEB-related studies [25,26]. Therefore, we examined the effects of trehalose on the progression of adenine-induced renal dysfunction and tubular injury in mice (Fig. 4). Our data demonstrated that trehalose treatment for up to 6 weeks did not reduce the progression of adenine-induced renal dysfunction and tubular injury, so we speculated whether TFEB plays a pathogenic role in adenine-induced CKD. Besides, we noticed that trehalose did not further enhance TFEB-autophagy signalling in renal tubules, likely suggesting that TFEB-mediated autophagy signalling was rapidly and/or maximally activated in renal tubules at the early stage of adenine-induced renal damages. To verify our inference, we examined the different doses of trehalose on TFEB activation and subsequent kidney injury *in vivo* at the early stage. As shown in Supplementary Fig. 3**A-D**, the mice that received adenine diet plus trehalose (2 and 4 mg/g) showed more obvious weight loss and increased plasma BUN levels compared with the adenine diet-treated mice as early as 2 weeks. We examined the protein expression of TFEB and the fibrosis marker αSMA in the kidney and found that TFEB in the kidney of trehalose-treated (2 and 4 mg/g) mice was higher than that in the kidney of the mice fed with adenine diet alone. This increase was accompanied with the up-regulation of αSMA expression, suggesting that this detrimental effect was related to further
TFEB activation. Thus, TFEB activation could aggravate adenine-induced renal injury at the early stage. Moreover, autophagy activation induced tubular atrophy and decomposition to facilitate fibrosis [27,28]. By contrast, an anti-fibrotic role of autophagy in kidneys was also reported by rescuing the inhibition of the G2/M arrest in the cell cycle or by regulating the degradation of mature TGFβ1 or collagen I to prevent kidney fibrosis [5,29]. Therefore, the role of autophagy in renal tubular injury in non-adenine CKD models remains controversial. Nonetheless, our findings supported the view that TFEB-mediated autophagy was injurious rather than protective to renal tubular cells in adenine-induced CKD at least in the early stage.

The pathogenesis of fibrosis is a complex process that involves all cell types in the kidney. Amongst all the cell types, renal tubular epithelial cells play a central role in regulating interstitial fibrosis, especially in the progression from acute kidney injury to CKD [30]. Persistent damage and

parenchymal cell death result in the loss of cell membrane integrity and the unbounded liberation of the products of dying cells and pro-fibrogenic mediators into the extracellular space that facilitates the local activation of collagen producing mesenchymal cells, such as myofibroblasts [31,32]. Interestingly, we identified that the TFEB-autophagy pathway was primarily up-regulated in tubular epithelial cells. In this aspect, this study further explored a pathogenic role of TFEB in renal tubular epithelial cells through the induction of autophagic cell death. Autophagy is normally considered as a protective mechanism of cell survival under various conditions including nutrient deprivation and hypoxia. On the contrary, excessive stress causes detrimental autophagy that leads to cellular dysfunctions by destroying organelles, especially the mitochondria and endoplasmic reticulum, and induces cell death [33,34]. The present study found that trehalose treatment significantly induced the activation of TFEB-mediated autophagy and caused cell death of the epithelioid clone of kidney cells, which were markedly blocked by bafilomycin (Figs. 5 and 6). Bafilomycin serves as a specific inhibitor of V-ATPase in cells, functions in decreasing proton influx to lysosome and results in the inhibition of calcium release and the phosphatase calcineurin, which in turn inhibits the TFEB activity. In this part, we evaluated the effect of 3-MA and choloquine in trehalose-induced cell death and found that the addition of 3-MA and choloquine did not affect cell death. This result implied that they did not target this pathway. These data indicated that TFEB activation alone is sufficient to trigger autophagic cell death, contributing to renal tubular epithelial cell injury. In addition, adenine-induced renal injury involves in oxidative stress [35,36], so we also investigated whether TFEB activation could promote tubular cell oxidative stress injury induced by H_2O_2, as shown in Supplementary Fig. 3**E**. We observed that trehalose could further aggravate H_2O_2-induced cell viability reduction. Our results were consistent with previous findings that autophagic cell death contributes to apoptosis induced by pro-fibrotic stimuli, including TGFβ in renal tubular epithelial cells [2].

These findings suggested that the injurious role of TFEB in adenine-induced renal damages was associated with excessive autophagy, consequent cell death and inflammation in the renal epithelioid clone of kidney cells. Under certain renal pathological conditions (such as excessive adenine supplementation), the persistent activation of autophagy in renal tubular epithelial cells may exceed the need for self-protection and cause excessive autophagic cannibalism, which ultimately provoke cell death leading. Consequently, tubular cell decomposition of tubular and tubular atrophy occur.

Recent studies have highlighted a critical but controversial role of epithelial–mesenchymal–transition (EMT) in renal fibrosis during the development of CKD [37–39]. Myofibroblasts are the most important cell types that generate inflammatory cytokines and collagenous matrix, leading to scarring and organ failure. They are likely derived from epithelial cells through EMT. The renal tubules with EMT exhibit a decreased expression of the epithelial marker E-cadherin and increased expression of mesenchymal marker α-SMA, inflammatory cytokines and extracellular matrix proteins. Autophagy promotes EMT in renal tubular epithelial cells [2,40,41]. However, the present study demonstrated that trehalose induced a decrease in the α-SMA expression (data not shown) and an increase in the IL-6 expression in the cultured epithelioid clone of kidney cells. Thus, our present data did not favour the role of TFEB activation in promoting EMT. Instead, IL-6 in epithelial cells might participate in renal fibrosis by activating local myofibroblasts to generate collagen I and expedite tubulointerstitial fibrosis. Our findings supported the view that adenine diet-induced tubulointerstitial inflammation and fibrosis were most likely due to inflammatory cell infiltration and myofibroblast activation secondary to TFEB-mediated death and decomposition of tubular epithelial cells.

In conclusion, our results indicated that TFEB-mediated autophagy activation contributed to an increased in cell death in adenine diet- induced renal injury. TFEB-mediated autophagy signalling was robustly up-regulated, particularly in the tubular epithelium, which was accompanied with tubulointerstitial inflammation and fibrosis. In the cultured epithelioid clone of the normal rat kidney cells, the TFEB activation by trehalose markedly induced the expression of autophagy- related genes, cell death and inflammation. These findings indicated that the induction of TFEB-mediated autophagy and autophagic cell death might contribute to tubular decomposition and fibrosis in CKD associated with a high purine diet. Strategies targeting TFEB expression showed potential for application in reducing renal inflammation and fibrosis to prevent CKD progression.

Compliance with ethical standards

All animal studies have been approved by the Animal Ethics Committee of Guangzhou University of Chinese Medicine (NO. 20190331042). Kidney tissue samples from the Department of Nephrology of Wuhan Union Hospital were collected after obtaining patient informed consent, all the human studies were approved by the ethics committee of Wuhan Union Hospital in China (NO. 81522010), and conformed with the Helsinki Declaration of 1975.

Declaration of Competing Interest

The authors declare that they have no conflict of interests.

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Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.biopha.2020.110949.

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